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FISH GILL IONIC TRANSPORT: METHODS AND MODELS

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Introduction

Because fishes, like all aquatic vertebrates except the marine hagfishes, maintain the Na^+ and Cl^- content of their body fluids distinctly different from either their freshwater or marine environment, they face a constant net movement of salts and water* across their permeable membrane (predominantly the branchial epithelium). Thus, freshwater fishes (which are hyperosmotic to the medium) presumably face a net loss of NaCl and net influx of water. The reverse is presumably true for the hypo-osmotic marine fishes, *i.e.* they face a net gain of NaCl and loss of water. The general mechanisms which fishes utilize to balance these net salt and water movements were first outlined by Homer Smith (1930) and have been more recently reviewed rather extensively (Potts and Parry, 1964; Maetz, 1974; Kirschner, 1977, 1979; Evans, 1979, 1980a). In the past few years it has become increasingly obvious that rather complex, but quite intriguing, mechanisms of ion transport are resident in the epithelium of at least the teleost fish gill** (Maetz and Bornancin, 1975; Maetz *et al.*, 1976; Kirschner, 1977, 1979, 1980; Potts, 1977; Evans, 1979, 1980b, 1982a).

It is not the aim of the present review to carefully re-examine the data which have been discussed in these reviews. Instead, we propose to examine some recent techniques which have been employed to attempt to more carefully delineate the mechanisms of fish branchial ionic transport. In the process we intend to describe what, and what not, these techniques can tell us about this system as well as what we think we know about the various ionic transport mechanisms, and where we think we should be heading in the future.

Since many of the techniques have been developed in the past 20 years to avoid some of the problems of *in vivo* studies, it is appropriate to begin with a description of whole animal kinetic and electrochemical studies.

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Abbreviations: IPHP, isolated, perfused head preparation; TAP, triaminopyrimidine; TEP, transepithelial potential.

* Since the elasmobranch fishes (sharks, skates, *etc.*) maintain isotonicity to sea water *via* the retention of urea, they are an exception to this statement about net movements of water. However, it is important to note that their body fluids contain significantly less NaCl than sea water, so they still face net influxes of these ions in sea water (see Evans, 1979, for a more complete examination of elasmobranch osmoregulation).

** Henceforth we will be dealing with teleost fish, with only slight reference to the elasmobranchs, until the last section of this review.

The Intact Animal

Investigations of possible mechanisms of gill transport actually started when Krogh (1939) demonstrated that the head end of goldfish (*Carassius auratus*) was able to extract Na^+ and Cl^- from solutions independently of each other. He proposed that, since the uptake mechanisms were parallel but uncoupled, there were probably ionic exchange systems involved to maintain some semblance of electroneutrality. He suggested that the Na^+ uptake might be coupled to NH_4^+ extrusion and that Cl^- uptake might be coupled to HCO_3^- efflux. In an early study utilizing radioisotopes Maetz and Garcia Romeu (1964) supported Krogh's proposition by demonstrating that Na^+ and Cl^- uptake could be stimulated, independently of each other, by injecting, respectively, NH_4^+ or HCO_3^- into the blood of the goldfish. Addition of the same substances to the external medium inhibited Na^+ and Cl^- uptake, respectively. Since the uptakes could be measured with ^{22}Na and ^{36}Cl, this study represented a significant step forward from earlier studies which relied on chemical analysis to monitor net fluxes. Nevertheless it also demonstrates at least two pitfalls of the majority of whole-animal studies, even to the present time. Na^+ and Cl^- are ions and their movements are therefore affected by electrical potentials as well as chemical gradients. Therefore it is possible that an experimental manipulation (in this case ionic substitution) which results in an alteration of the movement of an ion produces this change, not through a direct effect on some sort of ionic exchange system, but by altering the electrical gradient (transepithelial potential, TEP) across the epithelium in question. In other words, in the Maetz and Garcia Romeu (1964) experiments, it is possible (for example) that injection of $(NH_4)_2SO_4$ altered some TEP across the fish, made the blood more electronegative (relative to the fresh water) and thereby stimulated a passive uptake of Na^+. Corresponding arguments could be applied to the effects of $KHCO_3$ injections on Cl^- uptake and the effects of external additions of either substance on Na^+ and Cl^- uptake. Thus, firm statements about chemical *vs* electrical couplings simply cannot be made without concomitant measurements of the TEP.

The other pitfall of whole-animal studies (as demonstrated by the early work of Maetz and Garcia Romeu, 1964) is that specific alteration of the composition of the blood of intact animals is nearly impossible. For example the injection of $(NH_4)_2SO_4$ into the blood could have lowered the pH of the blood and thereby stimulated a Na^+/H^+ rather than Na^+/NH_4^+ exchange as proposed by the authors. Kerstetter *et al.* (1970) noted this potential and found that stimulation of Na^+ uptake (produced by increasing the external Na^+ concentrations) was correlated with a stimulation of acid efflux, rather than ammonia efflux. In addition, in this study, TEPs were monitored and shown to be insufficient to account for the increase in flux of either Na^+ or acid. Interestingly, the study by Kerstetter *et al.* (1970) demonstrates another drawback of whole-animal studies. They injected the carbonic anhydrase inhibitor acetazolamide into trout and found that both Na^+ uptake and acid efflux was inhibited. While these data could support the proposition that Na^+/H^+ exhange is present and limited by the production of protons by the hydration of CO_2 in the branchial cells, the fall in both fluxes could have been secondary to cardiovascular effects of the injected drug. That ammonia efflux did not change significantly during the same treatment argues against general cardiovascular effects, but it is possible that ammonia efflux is *via* a pathway which is relatively unaffected by cardiovascular changes. The unfortunate fact is that whole-animal studies do not allow the separation of cardiovascular from epithelial effects.

Thus, attempts to manipulate blood ionic concentrations, injection of potentially cardiovascular-active drugs, and lack of monitoring of the TEP present pitfalls which could bring into question the conclusions of many whole-animal studies. Some of these problems can be avoided by using externally applied drugs or by monitoring the efflux of an ionic species when ionic substitutions are made in the external medium. If one assumes that external addition of drugs or ionic substitutions do not have cardiovascular effects, and one monitors the TEP, many of the problems of earlier studies can be avoided. For example, we have recently found (Evans, 1977, 1982b; Evans *et al.*, 1979) that various species of marine teleost and elasmobranch fishes excrete ammonia and H^+, and that approximately 50% of the ammonia efflux and 100% of the H^+ efflux is dependent on external Na^+. Measurements of the TEP indicate that the coupling is not electrical. It is interesting to note that the excretion of ammonia from intact freshwater fishes is relatively unaffected by the removal of external Na^+ (deVooys, 1968; Kerstetter *et al.*, 1970; Maetz, 1973). Unfortunately, only Kerstetter *et al.* (1970) monitored the TEP, and they found that TEP changes were insufficient to account for any flux changes, or lack thereof.

The data from intact fish on Cl^-/HCO_3^- exchange is more sparse, but less equivocal. Dejours (1969) found that when the external medium of a goldfish was changed from NaCl to Na_2SO_4, CO_2 excretion fell to zero and was restimulated when the fish was again placed into NaCl solutions. In addition, DeRenzis and Maetz (1973) demonstrated a good correlation between the uptake of Cl^- and the net excretion of base by the goldfish, and DeRenzis (1975) found that addition of thiocyanate to the external bath inhibited Cl^- uptake and base excretion. Unfortunately, only in the latter study were TEPs measured, but they could not account for the effect.

In most cases whole-animal studies treat the branchial transporting cells as "black boxes" and cannot separate events taking place on the basolateral *vs* apical surfaces of the cells. For example, the fact that removal of external Na^+ inhibits 50% or less of the ammonia efflux from marine fish supports the proposition (Evans, 1977) that Na^+/NH_4^+ exchange is taking place, but it does not, in itself, indicate the site of this ionic exchange system. Maetz and Garcia Romeu (1964) found that the carbonic anhydrase inhibitor acetazolamide inhibited both Na^+ and Cl^- uptake when injected into the blood of the goldfish. They proposed that both Na^+ and Cl^- uptake must therefore be limited by the production of H^+ (for the protonation of NH_3 which had been produced in the branchial cells) and HCO_3^-. Since Na^+ and Cl^- are taken up independently from extremely low salinities (in micromolar ranges in some cases), and probably exchanged for these intracellular electrolytes (see above), it seems most appropriate to propose that the ionic exchange systems are on the apical border of the transporting cell (Maetz and Garcia Romeu, 1964; Kirschner, 1977, 1979). This model (Fig. 1) is supported by the finding that amiloride (which is known to inhibit uptake of Na^+ in a wide variety of tissues (Cuthbert *et al.*, 1979)) also inhibits both ammonia and acid efflux from fish (Kirschner *et al.*, 1973).

The potential problems of whole-animal studies are especially evident when one examines the history of the study of Na^+ and Cl^- extrusion mechanisms in marine species. A more extensive discussion of this subject was presented elsewhere (Evans, 1979). In the late 1960's it was found that the marine teleost branchial epithelium contained significant quantities of the enzyme (Na^+-K^+ activated ATPase) which mediated Na^+/K^+ exchange in a variety of tissues, and that these enzyme activity levels were lower in freshwater species and after freshwater adaptation of euryhaline

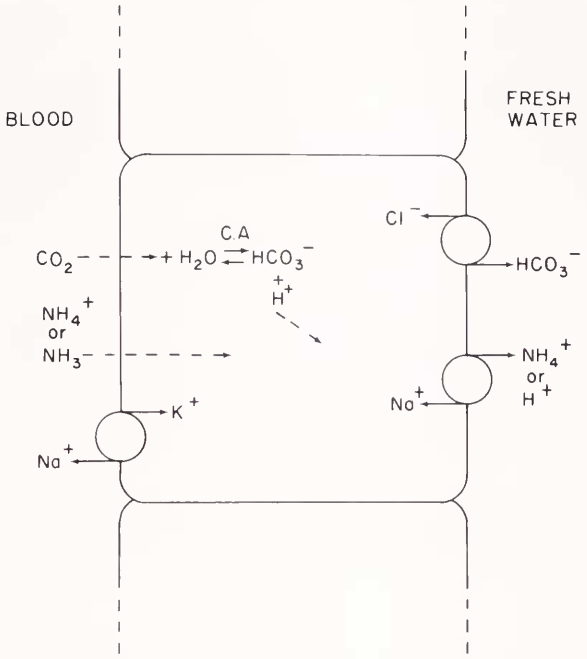

FIGURE 1. Tentative model for mechanisms of Na^+ and Cl^- uptake by the branchial epithelium of freshwater fishes. Redrawn from Maetz and Garcia Romeu (1964). See text for details, supporting evidence, and additions.

species (Epstein *et al.*, 1967). Soon thereafter it was discovered that the efflux of radiosodium from the eel, *Anguilla anguilla*, was sensitive to the external (seawater) concentration of K^+ (Maetz, 1969). Thus, it appeared that the seawater fish gill, like so many other tissues, extruded unwanted Na^+ in exchange for seawater K^+, utilizing the enzyme Na^+-K^+ activated ATPase (Maetz, 1971).

This model was strengthened by the finding that another species of marine teleost (the fat sleeper, *Dormitator maculatus*) also possessed a K^+-sensive Na^+ efflux, with a K^+ sensitivity (delineated by the K_m = 2 m*M* K^+) identical to that of the Na^+-K^+ activated ATPase extracted from the gill tissue (Evans *et al.*, 1973). In addition, the time course of activation of the enzyme was identical to the time course of activation of the K^+ sensitive Na^+ efflux when this species was transferred from fresh water to sea water (Evans and Mallery, 1975). However pleasant this model for Na^+ extrusion was, it rapidly became apparent that the system was much more complex. These initial studies neglected to measure the TEP during these ionic substitutions. Indeed, earlier studies (House, 1963; Evans, 1969) had demonstrated that the TEP across two species of marine teleosts was nearly identical to the equilibrium potential for Na^+, *i.e.*, Na^+ was possibly in passive equilibrium across the fish gill because the prevailing chemical gradient favoring net diffusional gain was balanced by a blood-positive (to sea water) electrical potential of sufficient magnitude to balance the chemical gradient. Thus, these data indicated that the net salt gain in sea water was not NaCl, but only Cl^- because Na^+ was in electrochemical equilibrium. This idea has been at least partially substantiated by more recent whole-animal TEP determinations; however, it has been found that some

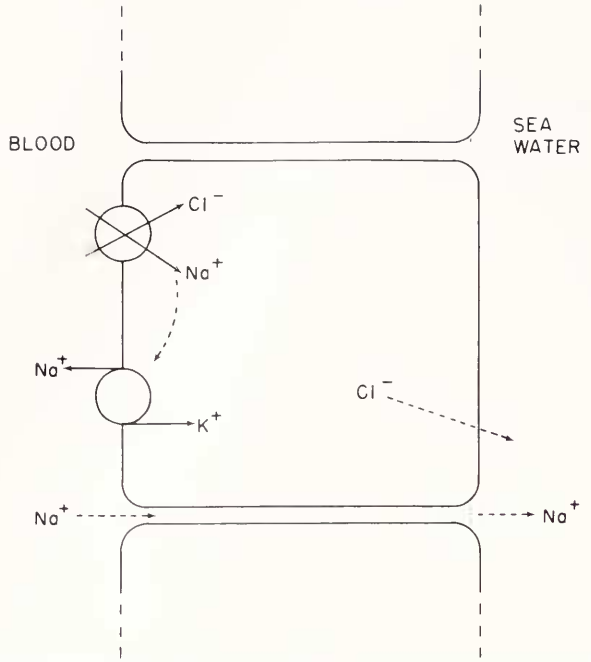

FIGURE 2. Current model for the mechanisms of Na^+ and Cl^- extrusion by the branchial epithelium of marine fishes. Redrawn from Silva *et al.* (1977). See text for details and supporting evidence.

species apparently maintain TEPs distinctively below the equilibrium potential for Na^+ (see reviews by Kirschner, 1979, 1980, and Evans, 1980b). If Na^+ is in electrochemical equilibrium then the ionic substitution experiments which indicated that Na^+/K^+ exchange may be taking place may also possibly be explained by TEP changes. This has proved to be the case in some species (Potts and Eddy, 1973; Kirschner *et al.*, 1974) but not in others (Evans, 1975; Maetz and Pic, 1975; Evans and Cooper, 1976). In addition, Na^+/Na^+ exchange diffusion which was first described by Motais *et al.* (1966) has now been shown to be a TEP effect in some species (Potts and Eddy, 1973; Kirschner *et al.*, 1974) but not others (Evans, 1975; Maetz and Pic, 1975; Evans and Cooper, 1976).

Thus, whole-animal studies on the mechanisms for salt extrusion in sea water have left us with the rather unsatisfying conclusion that some animals may be extruding net amounts of Na^+ and Cl^- and others may only need to extrude Cl^- (the TEP of all marine teleosts examined to date is distinctly different from the equilibrium potential for Cl^-; Evans, 1980b). Studies of the mechanisms of Cl^- extrusion by whole animals have indicated that it is sensitive to the external K^+ concentration (Epstein *et al.*, 1973) and to the external HCO_3^- but not OH^- concentration (Kormanik and Evans, 1979), and inhibited by injection of thiocyanate (Epstein *et al.*, 1973). In addition, both Na^+ and Cl^- efflux are inhibited by injection of the Na^+-K^+ activated ATPase inhibitor ouabain into the blood of the eel, *Anguilla rostrata* (Silva *et al.*, 1977). Since it had been shown that Na^+-K^+ activated ATPase is actually located on the basolateral plasma membranes (Karnaky *et al.*, 1976), these authors proposed that, like many other tissues (Frizell *et al.*, 1979), the marine teleost gill epithelium secretes Cl^- *via* a basolateral co-

transport of Na^+ and Cl^- (energized by the movement of Na^+ down its electrochemical gradient, which is maintained by Na^+-K^+ activated ATPase), followed by movement of Cl^- down its electrochemical gradient from the cell to the sea water. Na^+ is maintained in electrochemical equilibrium. This model (Fig. 2) certainly goes far to explain most of the present data from intact animals, but, of course, does not explain Na^+ extrusion by fish which have been shown to maintain Na^+ out of electrochemical equilibrium (see review by Evans, 1980b). The sensitivity to external HCO_3^- (Kormanik and Evans, 1979) is also not explained by this model.

Unfortunately, the use of intact animals precludes the most obvious experiments to test this interesting model. Ouabain is a potent cardiovascular agent and, indeed, Silva *et al.* (1977) did find that even the efflux of tritiated water from *A. rostrata* declined by some 40% after the injection of sufficient ouabain to produce a plasma concentration of 2.5×10^{-6} *M*. This presumably represented some sort of alteration in blood flow through the branchial vasculature, which in theory could have had a more pronounced effect of the efflux of both Na^+ and Cl^- than tritiated water. Thus, the fact that ouabain treatment inhibited Na^+ and Cl^- efflux by 90% does not necessarily prove a direct effect on a basolateral uptake, dependent upon a functioning Na^+/K^+ exchange. In addition, one cannot specifically remove blood Na^+ to examine the effect on Cl^- efflux (according to the Silva model, it would decline significantly).

In the past few years, whole-animal studies have been utilized to demonstrate that the Na^+/H^+ or NH_4^+ exchange which characterizes freshwater fish ion regulation is also present in marine species secondary to the needs of nitrogen and acid extrusion (see above). In fact we have found that the marine hagfish also possesses these ionic exchange systems (Evans, 1980a). Since hagfish have never entered fresh water (Hardisty, 1979) it appears that Na^+/H^+ or NH_4^+ exchange came about before the vertebrates entered fresh water, as an acid and nitrogen excretory device, rather than as an ionoregulatory device adaptive to freshwater existence. The presence of this system in marine species is therefore an indication of an ancient marine invention rather than a hold-over from a former existence in fresh water, as was formerly proposed (Evans, 1975).

Intact-animal studies have advanced our knowledge of fish branchial ion transport systems considerably in the past 20 years, but the limitations on the manipulation of intact animals has restricted the approaches to specific questions with somewhat limited answers. While the use of intact animals ensures (in theory) that proper perfusion and irrigation of the branchial epithelium is taking place, and that neural and hormonal inputs are present, it also ensures that substantial alterations in blood ionic components cannot be made, and that injection of known ionic transport inhibitors may induce secondary changes *via*, for instance, cardiovascular changes. Moreover, it does not allow one to separate transport steps at the basolateral *vs* apical borders of the transporting cells. In addition, the specter of stress with concomitant neuroendocrine changes is always present. For example, our finding that in both a marine teleost and marine elasmobranch replacement of the external sea water with Na^+-free artificial sea water (choline as the impermeant cation) resulted in cessation of net extrusion of H^+ and apparent extrusion of base leads us to believe that branchial Cl^-/HCO_3^- exchange may be present, but usually "hidden" behind Na^+/H^+ exchange, especially under hypercapnic conditions (Evans, 1982b). We tested for Cl^-/HCO_3^- exchange by injecting a bicarbonate load into both species with the expectation that we could stimulate net base excretion. However, in both cases (Evans, unpublished observations) we found that

injection of a base load stimulated net H^+ extrusion rather than net base extrusion, secondary, presumably, to a stress response. In fact injection of only Ringer's solution results in a net efflux of H^+. Thus, the stress response (despite the use of anesthetic) complicates an investigation of Cl^-/HCO_3^- exchange in intact animals.

Because of these problems, various *in vitro* approaches have been made to the study of fish gill transport in the past few years. We will start with perfusion of the head end since this technique was actually orginated in the 1930's.

The Isolated, Perfused Head Preparation

Keys (1931a) was the first to describe a fish preparation in which both the serosal and mucosal solutions bathing the branchial epithelium could be controlled. In this so-called "heart-gill" preparation (utilizing the eel, *Anguilla anguilla*) teleost Ringer's solution was perfused into the hepatic vein, and was pumped *via* the intact, beating heart to the gills. External irrigation of the gills with a small volume of fresh water was accomplished by pumping the water through a tube inserted into the mouth of the animal. Utilizing this preparation, the first *in vitro* experiments on branchial hemodynamic and active chloride transport mechanisms were described (Keys, 1931a,b; Bateman and Keys, 1932; Keys and Bateman, 1932; Keys and Wilmer, 1932). Thirty years later, the advent of isotopic tracers allowed a revitalization of the heart-gill preparation to attempt to define more clearly the NaCl movements across the gills (Tosteson *et al.*, 1962; Kirschner, 1969). The latter study modified the "heart-gill" preparation of the eel so that Ringer's was perfused into the ventral aorta *via* a pulsatile pump, thereby bypassing the heart. These pump-perfused gills appeared to be much more permeable to Na^+ than the heart-gill or *in vivo* systems also tested (Kirschner, 1969). By decreasing perfusate temperature, deterioration of ionic fluxes was reduced, but gill resistance still increased.

The utilization of the "heart-gill" preparation had one major undesirable attribute: the direct effects of various hemodynamic agents on the branchial vascular (*e.g.* epinephrine) could not be separated from their effects on the heart itself. The isolated, perfused head preparation (IPHP) of the trout, *Salmo gairdneri*, was developed by Payan and Matty (1975) and appeared to be much more viable as a tool for the study of osmoregulatory (and hemodynamic) parameters of the gills.

Briefly, the IPHP is prepared by decapitation of the fish posterior to the opercular openings after heparinizing and anesthetizing the animal. Cannulas are inserted into the ventral aorta proximal to the heart, and into the mouth. The preparation is then placed in a chamber which allowed the separation of irrigation fluid pumped over the gills from the efferent perfusate draining from the dorsal aorta and the open body cavity. Perfusion is accomplished either gravimetrically or by a peristaltic pump. Afferent flow rate or perfusion pressure is measured *via* a drop counter or pressure transducer connected to the perfusion line. In some preparations the dorsal aorta is cannulated, thus allowing the partitioning of the efferent perfusate into dorsal arterial and "venous" components (Girard and Payan, 1976; Claiborne and Evans, 1980).

This partitioning of respiratory and venous flows is possible since the blood leaving the respiratory lamellae in the gill may return *via* efferent filamental and branchial arteries to the dorsal aorta or be channelled through contractile anastomoses between the efferent filamental artery and the central venous sinus of the filament to the venous circulation (Fig. 3). In some species prelamellar anastomoses are also found (Boland and Olson, 1979) between the afferent filamental artery and the central vein of the filament, but these anastomoses are smaller and less

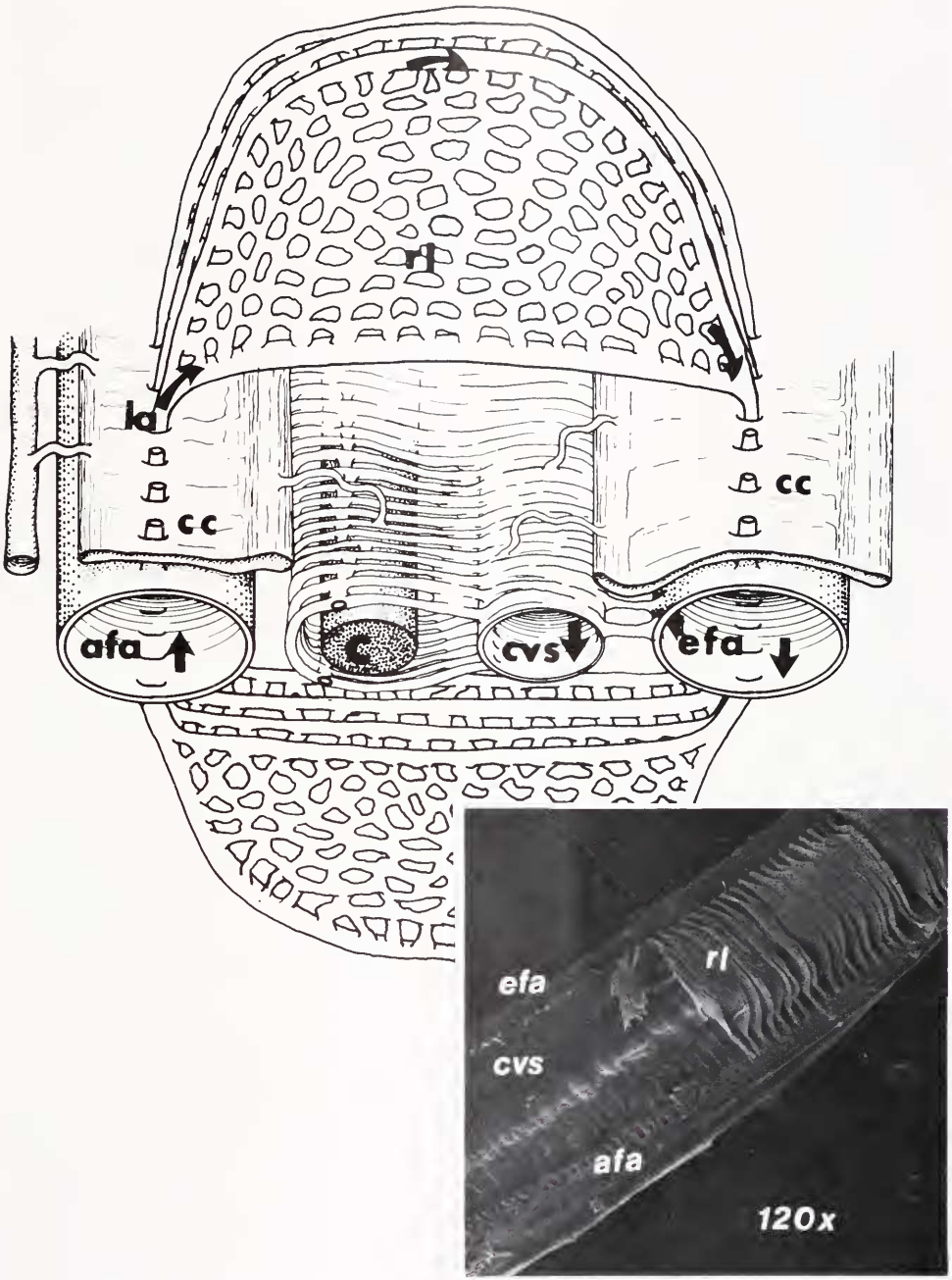

FIGURE 3. Filamental circulation of the gill of the snapper, *Lutjanus gresius*. Blood flows distally in the filament in the afferent filamental artery (afa) which leads to the respiratory lamellae (rl) *via* the lamellar arterioles (la). Blood leaves the lamellae in the efferent filamental artery (efa). Regular anastomoses between the efferent filamental artery and the central venous sinus (cvs) supply the extensive venous network surrounding the filamental cartilage (C). Companion nutritive vessels (cc) overlay both the afferent and efferent filamental arteries and connect to the central venous network *via* irregular anastomoses.

numerous than the postlamellar connections, and for that reason do not appear to form an effective bypass circuit around the lamellae (Farrell, 1980).

Since its inception, the IPHP has been used in a variety of investigations of gill hemodynamics (see Claiborne and Evans, 1980) and even brain metabolism (Callard *et al.*, 1981). We need concern ourselves only with the ionic transport studies.

Girard and Payan (1980) have recently reviewed their studies on the IPHP of the rainbow trout, *Salmo gairdneri*, which have demonstrated that the head is capable of carrying out Na^+/NH_4^+ exchange, both in fresh water (Payan, 1978) and in sea water (Payan and Girard, 1978). Unfortunately, no data have been published on the coupling of Na^+ influx to H^+ efflux or Cl^- influx to HCO_3^- efflux, despite the fact that both exchange systems have been described in the intact trout (Kerstetter *et al.*, 1970; Kerstetter and Kirschner, 1972). It is interesting to note that while Payan (1978) demonstrated a 1:1 stoichiometry for Na^+ and influx *vs* NH_4^+ efflux, a considerable ammonia efflux (approximately 70%) continued in the absence of Na^+ in the external bath, indicating clearly that the majority of the ammonia efflux is not coupled to external Na^+. These data corroborate our finding (Evans, 1977) that 50% or less of the ammonia efflux from intact marine species is dependent upon seawater Na^+. By examining the partitioning of the postlamellar (see Laurent and Dunel, 1980 for a review of fish gill morphology) perfusate flows into dorsal aorta *vs* "venous" flows, Girard and Payan (1977a) were able to demonstrate that all of the Na^+ and Cl^- influx was across the lamellar epithelium, contrary to the situation in the perfused head of the seawater-adapted trout where a significant portion of the influx is across the filamental surfaces, presumably interlamellar (Girard and Payan, 1977b). Since this lamellar Na^+ influx in the freshwater-adapted head displays the characteristics of Na^+/NH_4^+ exchange (*i.e.* is sensitive to perfusate NH_4^+ concentrations; Payan, 1978) it appears that the active transport step for at least freshwater Na^+ balance is in the lamellar epithelium, rather than the so-called "chloride cells" of the filamental epithelium. This is the first, and only, evidence that we have that ionic extraction by freshwater fish may actually not involve the "Cl cells," which are generally thought to be the sites of active salt transport in both freshwater and seawater fish (see below). It is important to note that the influxes of both Na^+ and Cl^- displayed by the perfused trout head were only 20–30% of those found *in vivo* (Girard and Payan, 1977a); however, 10^{-5} *M* epinephrine stimulated Na^+ influx to some 130% of *in vivo*, while the Cl^- influx remained unchanged (Girard and Payan, 1977a).

The IPHP has also been utilized to examine more carefully the cellular localization of the Na^+/NH_4^+ exchange mechanism. Payan *et al.* (1975) demonstrated that addition of ouabain inhibited both Na^+ influx and ammonia efflux from perfused freshwater trout heads. However, Payan (1978) proposed that this inhibition was secondary to a primary, apical Na^+/NH_4^+ exchange which was sensitive to intracellular Na^+ concentrations (which were maintained by basolateral Na^+-K^+ ATPase). This model was based upon his finding that acetazolamide added to the perfusate inhibited ammonia clearance, as did amiloride added to the irrigation fluid (fresh water). More critically, he found that reducing the NH_3 concentration of the perfusate by approximately 10-fold (by reducing the pH by 1 pH unit) inhibited the excretion of ammonia by some 85%. It also inhibited sodium influx by about 60% (Payan, 1978). He therefore proposed that ammonia entered the cell as NH_3, was protonated *via* the hydration of CO_2 *via* carbonic anhydrase, and was excreted at the apical surface in exchange for Na^+ in the fresh water—the model first proposed by Maetz and Garcia Romeu in 1964 for intact fish (see above). We

have recently approached the same problem with the IPHP of two marine teleost fishes (*Myoxocephalus octodecimspinosus*, the longhorned sculpin; and *Opsanus beta*, the gulf toadfish) and found that increasing the perfusate NH_3 concentration (by increasing the pH) did not stimulate ammonia efflux; however, increasing only the NH_4^+ concentration (by increasing the ammonia concentration, while reducing the pH) stimulated the ammonia efflux significantly (Goldstein *et al.*, 1982). Since only approximately 50% of the ammonia efflux is coupled to Na^+ in intact marine fishes (see above), it appears that a significant component of ammonia efflux from at least marine fish gills is *via* diffusion of NH_4^+ across the branchial epithelium. This may be *via* leaky "tight junctions" since the marine teleost gill has been shown to be quite leaky to cations and even large organic molecules (Karnaky, 1980). The proposition that NH_4^+ can diffuse across the marine teleost gill is supported by our earlier finding that addition of 200 m*M* NH_4Cl solutions to Na-free artificial sea water depolarized the TEP across the intact toadfish to the same extent as 200 m*M* NaCl (Evans, 1977). It is important to note that the ammonia efflux from the IPHP of both *O. beta* and *M. octodecimspinosus* is close to that found *in vivo* (Goldstein *et al.*, 1982). We have found that ouabain added to the Ringer's solution (containing 1 m*M* NH_4Cl) perfusing the IPHP of *O. beta* inhibited ammonia efflux by some 50%. This could have been an indirect effect (as proposed by Payan, 1978); however, we have also found that addition of K^+ to the perfusate inhibited ammonia efflux, indicating a direct interaction at the basolateral border. In addition, we found that neither ouabain nor K^+ produced hemodynamic effects sufficient to account for the observed inhibition of ammonia efflux (Claiborne *et al.*, 1982). We conclude that, at least in this species, Na^+/NH_4^+ exchange is basolateral, rather than apical, and running through the Na^+-K^+ activated ATPase. The NH_4^+ sensitivity of this enzyme is well documented (see below).

Since intact marine teleosts and elasmobranches have been shown to excrete H^+ in exchange for Na^+ (Evans *et al.*, 1979; Evans, 1982b) it would be of great interest to use an IPHP to examine this system in greater detail.

Girard (1976) used the IPHP of the seawater-adapted trout to examine various aspects of the extrusion of Na^+ and Cl^-. He found that the effluxes of Na^+ is near to that measured *in vivo* and that effluxes of both Na^+ and Cl^- were stimulated by addition of K^+ to the external medium; unfortunately he did not report TEPs so that one could separate chemical *vs* electrical coupling. Claiborne and Evans (1981) have recently shown that the IPHP of *M. octodecimspinosus* maintains a Na^+ efflux near *in vivo* levels, but a Cl^- efflux significantly below that found in the intact fish. The efflux of neither ion is affected by large alterations in the irrigation rate, but changes in perfusion rate (and therefore pressure) produce significant alteration in the Na^+ efflux, with no effect on the Cl^- efflux. This argues for separate pathways for the bulk of the Na^+ *vs* Cl^- efflux which supports the extrusion model of Silva *et al.* (1977; see above), but does not support the recent porposition (Sargent *et al.*, 1978; Kelly *et al.*, 1981) that NaCl is forced across the leaky "tight junctions" of the branchial epithelium by arterial blood presure.

Various direct tests of the "Silva model" are theoretically possible with the IPHP. Since the perfusate can be manipulated it would be of great interest to test the sensitivity of the Cl^- efflux to removal of Na^+ from the perfusate. This would be the most direct test of the proposed co-transport of Na^+ and Cl^- which is the core of this model. Unfortunately, the branchial vasculature of at least *M. octodecimspinosus* is quite sensitive to the choline used to replace the perfusate Na^+ and subsequent large increases in afferent pressure and Cl^- efflux obscure any

changes in Cl^- efflux which may have been produced by the lack of Na^+ (Claiborne and Evans, unpublished).

Kelly *et al.* (1981) have recently found that 10^{-4} *M* ouabain inhibits both the Na^+ and Cl^- efflux from the IPHP of the eel (*Anguilla anguilla*) by some 30–40% with no effect on the afferent perfusion pressure, or the efflux of tritiated water. Thus, in these experiments, one can be rather certain that the effect of ouabain was a direct one on some component of the transport system, rather than an indirect effect through hemodynamic changes.

To date, no report of a transepithelial potential measured across the gills of an IPHP has appeared in the literature. TEP changes across the branchial epithelium must be monitored concurrent with ion substitution or drug inhibition experiments (see above). Recently, we have found it possible to measure the TEP across the gills of the IPHP of *M. octodecimspinosus* in sea water. We found that the IPHP TEP was similar to that measured *in vivo*. Substitution of Na^+ or Cl^- with the appropriate impermeant ion in the external sea water resulted in large depolarizations when Na^+ was replaced, but no alterations were observed after Cl^- substitutions (Claiborne and Evans, 1981). These responses, observed both *in vivo* and *in vitro*, indicate that the gills of the IPHP (and the sculpin *in vivo*) are more permeable to Na^+ than to Cl^-, as shown in many other teleosts which possess a positive TEP (Evans, 1979).

While the IPHP enables the investigator to ask questions impossible using intact systems, it still presents some limitations. The majority of the studies of ion transport by the IPHP have utilized the trout head, which suffers from rather serious hemodynamic degradation in a short period of time. Girard (1976) found that the gill resistance increased by some 5-fold within 30 minutes and Wood (1974) found that relatively linear and stable pressure *vs* flow relationships were only possible if post-branchial efferent pressures were maintained by a column of irrigation solutions. To delay the hemodynamic degradation of the trout head, epinephrine has sometimes been added to the perfusate (Payan, 1978). However, it is clear that this hormone stimulates Na^+ uptake in fresh water and inhibits it in sea water (Girard, 1976; Payan, 1978; Shuttleworth, 1978). This hemodynamic degradation of the IPHP may be species specific since we have recently found that IPHPs of the sculpin, toadfish, and shark "pup" (*Squalus acanthias*) can maintain relatively consistant gill resistances for 3–8 hours (Claiborne and Evans, 1980; Oduleye *et al.*, unpublished results; Evans and Claiborne, 1982). In all three species the afferent pressures are at *in vivo* levels when the perfusion rate is in the same range as the *in vivo* cardiac output, despite the fact that postbranchial efferent resistances are near zero. It is obvious that other species should be examined.

Importantly, most of the IPHP studied to date maintain Na^+ and/or Cl^- fluxes significantly below *in vivo* levels (see above). In fact, in a recent study using the IPHP of *A. anguilla* the measured Na^+ and Cl^- fluxes were only 10% of the fluxes measured *in vivo* (compare Kelly *et al.*, 1981, with Epstein *et al.*, 1973). Whether the reduced effluxes found in some species are secondary to incomplete perfusion of the branchial vasculature or lack of stimulatory hormones normally found *in vivo* remains to be determined.

Probably the most important, and least often controlled, parameter of the IPHP is the ratio of the perfusate inflow to outflow. Most authors do not note this comparison which is a direct measure of the structural/hemodynamic integrity of the system. It should be obvious that even slight leakage of the perfusate either into the external medium or the head tissues will produce quite spurious determination

of ion flux rates. These leak pathways may not affect active pathways, but they may obscure the latter's importance or even presence in the total unidirectional flux as determined with radioisotopes. Losses of up to 30% of the perfusate during its transit of the gills has been reported by some investigators in personal communications. Again this may be species specific because we have found that the sculpin, toadfish, and dogfish shark "pup" maintain inflow:outflow ratios of approximately 1.0 (Claiborne and Evans, 1980; Oduleye *et al.*, unpublished results; Evans and Claiborne, 1982). In summary, present data indicate that the isolated, perfused head preparation may allow a more critical dissection of the mechanisms of NaCl transport by the fish branchial epithelium than is possible with *in vivo* studies. It is important to note that in most instances published IPHP studies have corroborated the findings of earlier studies using intact animals, despite the fact that many of the preparations (especially those utilizing the trout head) display significant degeneration of the hemodynamics of the branchial vasculature. It is clear that more species need to be investigated and that greater attention be paid to the ratio of the inflows:outflows and the TEPs maintained by the IPHP.

The Isolated, Perfused Gill Preparation

An alternative to the perfused head is the isolated, perfused gill, which has been used rather extensively in the past 15 years. Although methods vary slightly, generally isolated gills are prepared by initial perfusion of an anesthetized animal with Ringer's solution. When filaments are free of blood, individual arches are selected and removed. The afferent and efferent branchial arteries are cannulated, and the arch is placed in a well-stirred external bath. In early work a constant pressure reservoir provided afferent pressure, but more recently pulsatile flow generated by a pump has been employed. Efferent pressure is set by the height of the efferent cannula above the preparation.

Like the perfused head, the perfused gill preparation has been used extensively to investigate the hemodynamics of branchial circulation, but rather little to study gill ion transport. To a considerable extent this is apparently due to the isolated gill's ability to maintain reasonable hemodynamics (*e.g.* Bergman *et al.*, 1974; Holbert *et al.*, 1979) but inability to maintain proper irrigation. Unfortunately, even vigorous stirring of the irrigation bath apparently does not mimic the irrigation patterns found in the intact animal, or the perfused head. For example, Shuttleworth and Freeman (1974) described Na^+ and Cl^- effluxes from the perfused eel (*Anguilla dieffenbachii*) gills that were only 10–15% of those found in the intact fish, and Farmer and Evans (1981) have recently found that the efflux of Cl^- from perfused pinfish (*Lagodon rhomboides*) gills is 45% that of the intact fish. Nevertheless, the perfused gill has provided us with some information unavailable with other techniques. Shuttleworth *et al.* (1974) demonstrated that the TEP across the perfused marine flounder (*Platichthyes flesus*) gill was approximately 7 mV inside positive when the gill was perfused and irrigated with Ringer's solution. Addition of ouabain inhibited the TEP, indicating that salt extrusion was electrogenic, and that Na^+-K^+ activated ATPase played an important role. The finding of a substantial TEP when no chemical gradients existed across the gill epithelium demonstrated that the TEP across intact marine fish was probably a combination of electrogenic transport and differential ionic permeabilities. Studies with intact marine fish had suggested that the TEP was primarily the result of a much higher cation than anion permeability (Potts and Eddy, 1973; Kirschner *et al.*, 1974). More recent studies have lent support for the "Silva model" for coupled Na^+ and Cl^- transport

by the gill epithelium. Farmer and Evans (1981) have shown that the Cl^- efflux from the perfused pinfish gill is inhibited by removal of Na^+ from the perfusate, or addition of furosemide. Furosemide has been found to inhibit coupled Na^+ and Cl^- transport in a wide variety of epithelial tissues (Frizell *et al.*, 1979).

The perfused gill has been utilized to examine salt uptake by freshwater fish. Richards and Fromm (1970) found that addition of ouabain to the Ringer's solution perfusing the isolated trout gill inhibited the uptake of Na^+ and Shuttleworth and Freeman (1974) found that removal of K^+ from the perfusate inhibited Na^+ uptake by the eel gill. Both studies support the conclusion that basolateral Na^+/K^+ exchange (mediated *via* Na^+-K^+ activated ATPase) plays a role in Na^+ uptake in fresh water.

The Isolated Opercular Epithelium

An extensive literature indicates that the mitochondria-rich "chloride cell" of the fish gill epithelium plays an important role in osmoregulation (for an extensive review see *The Biology of the Chloride Cell*: Jean Maetz Memorial Symposium, *American Journal of Physiology* **238:** R141–R276, 1980). Quite recently a technique has been developed which has enabled a much more direct study of the biophysics of ion transport across this cell than has been possible with intact fish, or isolated heads or gills.

Burns and Copeland (1950) demonstrated that "chloride cells" are widely distributed throughout the head region of the killifish, *Fundulus heteroclitus*, but it was not until 1977 that it was shown that the opercular epithelium of this species possesses a cellular population which is 50–70% "chloride cells" whose cytology and ultrastructure is identical to the "chloride cells" in the gill epithelium (Fig. 4) (Karnaky *et al.*, 1976; Karnaky and Kinter, 1977). Thus, the opercular epithelium presented the unique opportunity to investigate the function of "chloride cells" on a flat epithelium, rather than on the extremely complex branchial epithelium. A flat epithelium can be dissected free and mounted in an "Ussing Chamber" which enables a strict thermodynamic approach to the electrical and chemical events of ion transport. In this way one can carefully control the ionic composition of both serosal and mucosal solutions bathing the tissue and measure net movements of ions quite accurately. In addition, any spontaneously generated electrical potentials can be measured and nulled (to quantify the short-circuit current), and resistances can be calculated. Since the original description of the opercular epithelium of *F. heteroclitus*, similar, "chloride cell"-rich tissues have been found in the operculum of *F. grandis* (Krasny and Evans, 1980) and *Sarotherodon mossambicus* (Foskett *et al.*, 1979) as well as the jaw epithelium of *Gillichthys mirabilis* (Marshall and Bern, 1980).

When the isolated opercular epithelium from seawater-adapted killifish is bathed bilaterally with a Ringer's solution having an ionic composition similar to *F. heteroclitus* plasma, a potential difference oriented serosa (blood) positive is generated (Degnan *et al.*, 1977; Karnaky *et al.*, 1977). Subsequent isotopic flux studies (Table I) showed that this potential difference was the result of the net transport of Cl^- outwards across the tissue, *i.e.* blood side to seawater side; there was no net transport of Na^+ across the epithelium (Degnan *et al.*, 1977; Karnaky *et al.*, 1977). These were the first unequivocal studies showing that killifish maintain ionic homeostasis in sea water by actively extruding chloride into the external milieu. Equivalancy between the short-circuit current and net Cl^- secretion has also been observed in the "chloride cell" containing opercular epithelia of *F. grandis*

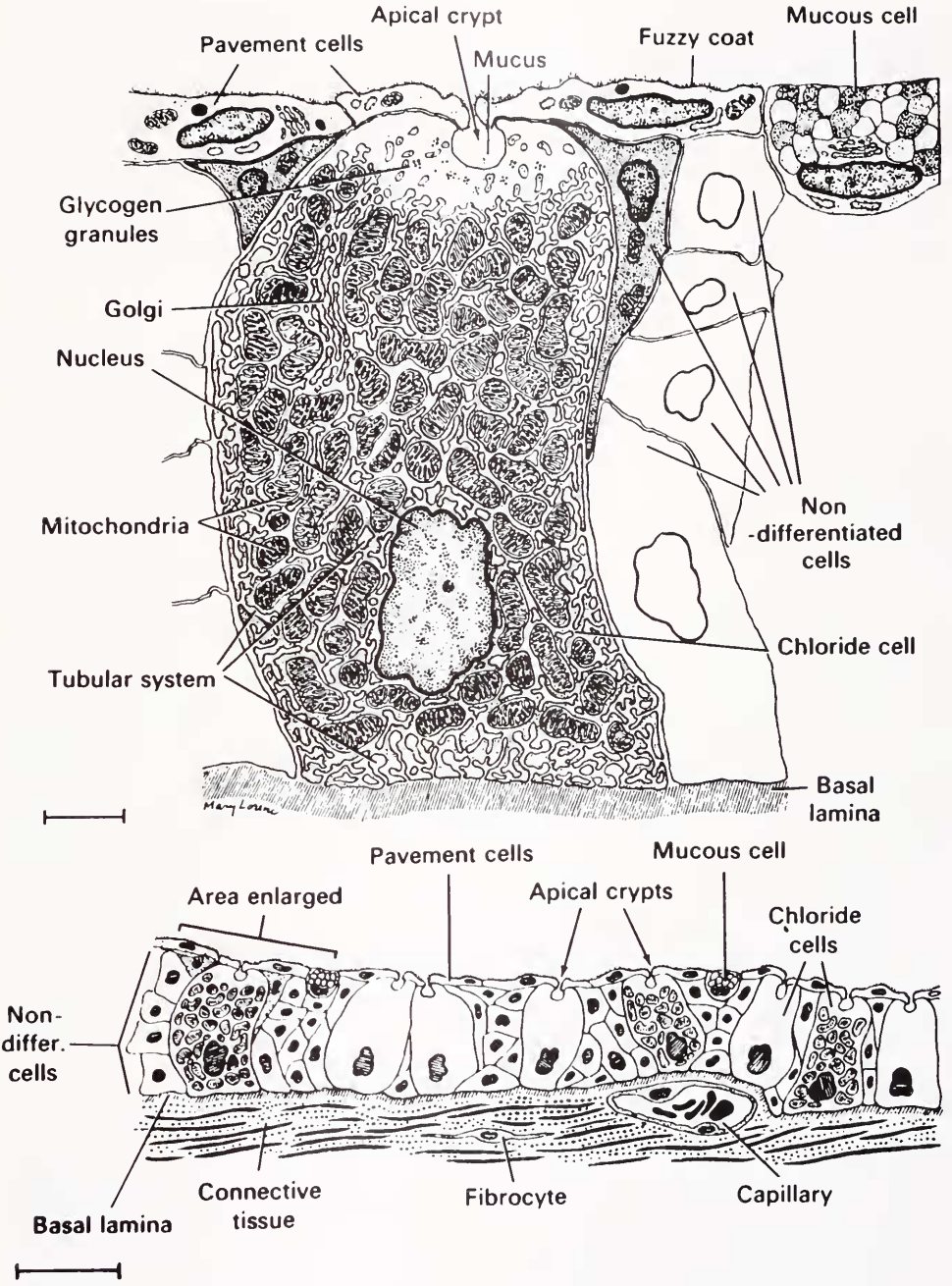

FIGURE 4. Schematic of the ultrastructure of a "chloride cell" (upper) and opercular epithelium (lower) from the opercular epithelium from *Fundulus heteroclitus*. In this tissue 50–70% of the cellular population is represented by "chloride cells" whose cytology is identical to that described for the branchial epithelium of teleosts. Reproduced with kind permission from Degnan *et al.* (1977). See text for details of the physiology of this opercular tissue. Scale is 20 μm.

TABLE I

Isotopic fluxes and electrical properties across the short-circuited opercular epithelia of seawater-adapted Fundulus heteroclitus *gassed with 95% oxygen, 5% carbon dioxide.*

	Efflux	Influx	Net Flux	SCC	PD
Cl	7.23 ± 2.13	2.86 ± 1.13	4.46 ± 1.09/119.6 ± 29.3	119.2 ± 22.9	12.6 ± 1.2
Na	2.63 ± 0.45	2.95 ± 0.26	−0.32 ± 0.62/−8.6 ± 16.5	74.4 ± 10.3	10.1 ± 1.5

N for fluxes is 8, N for electrical properties is 16. Fluxes in $\mu Eq \cdot cm^{-2} \cdot h^{-1}$, net fluxes in $\mu Eq \cdot cm^{-2} \cdot h^{-1}/\mu A \cdot cm^{-2}$. Short circuit current (SCC) in $\mu M \cdot cm^{-2}$ and potential difference (PD) in mV, serosa relative to mucosa. Data from Degnan *et al.* (1977). Note that the SCC is identical to the net influx of Cl with no net movements of Na.

(Krasny, 1981) and *Sarotherodon mossambicus* (Foskett *et al.*, 1979) and jaw epithelium of *Gillichthys mirabilis* (Marshall and Bern, 1980).

The transport mechanisms in "chloride cells" for chloride appears very similar to that found in most chloride-transporting epithelial types (Frizzell *et al.*, 1979; Frizzell and Duffey, 1980). Namely, Cl^- efflux is dependent upon the presence of Na^+ in the serosal medium (Degnan and Zadunaisky, 1980a, 1981; Mayer-Gostan and Maetz, 1980) and is blocked by the transport inhibitors furosemide or ouabain on the serosal side (Degnan *et al.*, 1977; Karnaky *et al.*, 1977; Mayer-Gostan and Maetz, 1980).

Studies utilizing the short-circuit current techniques made in conjunction with fluorescence microscopy techniques have provided direct evidence that the "chloride cell" is the "active" ionocyte involved in seawater teleost osmoregulation. DASPMI, a low toxicity, specific fluorescent stain for mitochondria in living cells (Bereiter-Hahn, 1976), has been used to stain "chloride cells" in the opercular epithelium of *F. heteroclitus* (Zadunaisky, 1979). Studies using this dye in the opercular epithelium of *F. heteroclitus* (Karnaky *et al.*, 1979) and the jaw skin epithelium of *Gillichthys mirabilis* (Marshall and Nishioka, 1980) have shown a linear correlation between "chloride cell" density and the magnitude of the short-circuit current. Similarly, Foskett *et al.* (1979) have shown that the increase in "chloride cell" density and size is correlated to the development of a short-circuit current in the opercular epithelium of *Sarotherodon* when the fish is acclimated to sea water. More recently, Foskett and Scheffey (1982) have found, using a vibrating probe technique, that current generated by the short-circuited opercular epithelium is directly over the "chloride cells". This is certainly the most definitive demonstration that the "chloride cells" are the site of electrogenic Cl^- transport across the fish branchial epithelium.

By studying the voltage dependency of the unidirectional flux of an ion across an epithelium one can predict the nature (conductive *versus* electroneutral) and the pathway (cellular *versus* paracellular) of ion flow (Frizzell and Schultz, 1972; Mandel and Curran, 1972). Results from studies made in opercular epithelia of *F. heteroclitus* (Degnan and Zadunaisky, 1980b) and *F. grandis* (Krasny, 1981) indicated that there were no significant differences between the predicted and measured fluxes for either the efflux or influx of Na^+, thus allowing the conclusion that the Na^+ fluxes in opercular epithelia are passive and traverse only one rate-limiting barrier. This rate-limiting barrier presumably is represented by the tight junctional complex between neighboring "chloride cell" (Sardet *et al.*, 1979; Ernst *et al.*, 1980) as is indicated from experiments with triaminopyrimidine (TAP). TAP, which blocks passive cation transport through the paracellular pathway in "leaky" epithelia (Moreno, 1975) reduces the Na^+ efflux 84.1%, while reducing the total

tissue conductance 77%, in the opercular epithelium of *F. heteroclitus* (Degnan and Zadunaisky, 1980b).

These results suggesting passive Na^+ movements as well as the results from studies made on the mechanism of chloride secretion in the opercular epithelium have provided strong direct evidence for the Silva *et al.* (1977) model for "chloride cell" function in seawater teleosts.

Whereas the isolated opercular epithelium from seawater-adapted teleosts has been used to define the ion transport properties of "chloride cells," the use of this preparation in the study of freshwater ion regulation is relatively uninvestigated. Although intact *F. heteroclitus* maintain ionic homeostasis in fresh water by extracting Na^+ and Cl^- from the environment (Maetz *et al.*, 1967; Potts and Evans, 1967), opercular epithelia from freshwater-adapted *F. heteroclitus* continue to secrete Cl^- (Degnan *et al.*, 1977). This may, in fact, be due to autoregulation of the apical membrane permeability to Cl^- induced by the exposure of freshwater opercular epithelia to a Ringer bathing media containing 142.5 m*M* Cl, *i.e.* chloride regulates its own membrane permeability (Ques-von Petery *et al.*, 1978). Evidence for this supposition can be found in experiments performed on seawater-adapted opercular epithelia where removal of Cl^- from the mucosal bathing media results in a decrease in tissue conductance and reduces the rate of Cl^- secretion (Degnan and Zadunaisky, 1980a). On the other hand, opercular epithelia, isolated from normally freshwater-occurring *Sarotherodon* (Foskett *et al.*, 1979) or from *F. heteroclitus* which had been chronically injected with the "freshwater" hormone prolactin (Mayer-Gostan and Zadunaisky, 1978), are characterized by low short-circuit currents and high electrical resistances. This might be expected since: 1) the active chloride secretory process is "turned off;" and 2) the proposed ionic uptake mechanisms for both Na^+ and Cl^- in the branchial epithelium of freshwater teleosts are, in fact, one for one electroneutral (electrically silent) exchanges: Na^+/H^+ and/or NH_4^+ and Cl^-/HCO_3^- (see above).

Although it has been suggested that the Na^+/H^+ or Na^+/NH_4^+ exchanger may be located in the pavement cells of the lamellae of the branchial epithelium (Girard and Payan, 1980), these cells are derived from the filamental epithelium (Morgan, 1974; Laurent and Dunel, 1980) and are identical to the pavement cells of the opercular epithelium as determined by thin section electron microscopy (Karnaky and Kinter, 1977; Ernst *et al.*, 1980) and freeze-fracture (Sardet *et al.*, 1979; Ernst *et al.*, 1980) techniques. Thus, the use of the isolated opercular epithelial preparation with the pH-stat technique may yield new and important information concerning the ionic mechanisms involved in acid-base balance in both freshwater and seawater teleosts.

In theory, the isolated opercular epithelium may provide us with a vehicle for studying intracellular ionic concentrations and basolateral *vs* apical transport events *via* microelectrodes, in a manner similar to that recently used for a variety of transporting epithelia (Frizell *et al.*, 1979). However, the complex geometry of the extensive basolateral tubular invaginations results in a relatively sparse cytoplasm which may hinder such determinations.

It is obvious that the isolated opercular epithelium has allowed substantial advances in the investigation of the biophysics of NaCl extrusion by a seawater-acclimated teleost. However, one must be cautious when extending these data to all marine teleosts, and especially those species which seem to maintain Na^+ out of electrochemical equilibrium. In addition, it remains to be seen if it will be useful for the investigation of other transport events such as Na^+/NH_4^+, Na^+/H^+, and Cl^-/HCO_3 exchange.

Isolation and Characterization of Transport ATPases

The foregoing demonstrates the central role of Na^+-K^+ activated ATPase in ion balance and nitrogen excretion by the teleost branchial epithelium. This subject has also been recently reviewed by Epstein *et al.* (1980), Karnaky (1980), and Towle (1981). The assay of enzymatic activity primarily in whole gill homogenates has been especially productive in assessing salinity adaptive changes. This approach may be biased by differences between biochemical techniques (homogenization time, temperature, pH detergents, *etc.*) of different laboratories, and changes in tissue protein levels which will bias specific activity measurements if microsomal fractions are used. Unfortunately, the biochemical isolation and characterization of this presumptive transport enzyme is rather rare. Isolation and purification is certainly the approach which ought to be more productive in defining the ionic parameters that this gill enzyme functions under.

Partial characterizations of branchial Na^+-K^+ activated ATPase have been published (Kamiya and Utida, 1969; Pfeiler and Kirschner, 1972; Giles and Vanstone, 1976; Ho and Chan, 1980), but the publications from Sargent's laboratory (Sargent and Thomson, 1974; Bell *et al.*, 1977; Bell and Sargent, 1979; Sargent *et al.*, 1980) present the most detailed analysis of the enzyme from the fish gill (the Atlantic eel, *Anguilla anguilla*). They have purified the Na^+-K^+ activated ATPase to a specific activity of approximately 400 $\mu M \cdot$ mg protein$^{-1} \cdot h^{-1}$, one to two orders of magnitude greater than that described by other authors (see Kirschner, 1980 for representative data). The enzyme, like that isolated from mammalian kidney and shark rectal gland (Dahl and Hokin, 1974; Schwartz *et al.*, 1975) is phosphorylated in the presence of Na^+ and Mg·ATP to produce a phosphoenzyme intermediate, which is dephosphorylated in the presence of K^+. Ouabain inhibits the dephosphorylation step and other cations, including NH_4^+, can substitute for K^+ at the dephosphorylation step, with varying affinities. Bell *et al.* (1977) found that the affinity of the purified enzyme for NH_4^+ was slightly less than for K^+, while Mallery (1979) found that partially purified enzyme from *O. beta* displayed a higher affinity for NH_4^+ than for K^+. It is interesting to note that this species displays a ouabain and K^+-sensitive ammonia efflux (Claiborne *et al.*, 1982). Unfortunately we have no data on the molecular weight or subunit structure of the fish branchial Na^+-K^+ activated ATPase.

We know even less about a putative anionic transport ATPase. Kerstetter and Kirschner (1974) described an ATPase fraction from trout branchial tissue which was stimulated by HCO_3^- and inhibited by thiocyanate. Both the enzyme and Cl^- influx was inhibited by thiocynate, which is especially surprising considering that the enzyme was inhibited, rather than stimulated by the addition of Cl^- to the incubation medium. Importantly, comparison with succinic dehydrogenase activity as a mitochondrial marker indicated that the HCO_3^--stimulated ATPase was in both mitochondrial and microsomal fractions. More recently DeRenzis and Bornancin (1977) and Bornancin *et al.* (1980) have described a microsomal ATPase which is stimulated by both HCO_3^- and Cl^-, and inhibited by thiocyanate. Importantly, they have shown that this fraction is not contaminated by mitochondrial anion ATPase. They suggest that the enzyme is important in Cl^- balance and acid/base regulation in fresh water since previous studies (DeRenzis, 1975) had shown that Cl^- influx was correlated with base (presumably HCO_3^-) excretion and inhibited by thiocyanate (see above). It is unclear if the enzyme functions in Cl^- transport in the marine teleosts. Kormanik and Evans (1979) have described an external HCO_3^--sensitive efflux of Cl^- from *O. beta* in sea water, and Epstein *et*

al. (1973) did find that injection of thiocyanate inhibited Cl^- efflux from seawater eels. However, the fact that the activity of the Cl^--HCO_3^- activated ATPase did not change upon acclimation to sea water (Kerstetter and Kirschner, 1974; Bornancin *et al.*, 1980), despite a significant difference in the rate of Cl^- transport across the freshwater *vs* seawater gill (Evans, 1979) suggests that its major role may be in the freshwater environment. Indeed, the Silva model for NaCl extrusion by marine teleosts (Silva *et al.*, 1977) suggests that Cl^- exits the Cl cell down electrochemical gradients across the apical surface of the "chloride cell," rather than *via* Cl^-/HCO_3^- exchange. The latter cannot be ruled out at present however. It is clear that a more detailed investigation of the role of Cl^--HCO_3^- activated ATPase in fish ion regulation is needed.

The Elasmobranchs

Interest in the rectal gland has nearly stifled investigation of the elasmobranch branchial epithelium. However, the ability of some species to tolerate sea water for prolonged periods after removal of the rectal gland, and the recent finding that Na^+/NH_4^+ and Na^+/H^+ ionic exchanges are resident in the elasmobranch branchial epithelium (Evans, *et al.*, 1979; Evans, 1982b) suggest that the gills may play some role in salt extrusion (see Evans, 1979 for a more complete discussion of the role of the rectal gland *vs* branchial epithelium).

To a considerable extent the paucity of data on the mechanisms of ionic transport across the elasmobranch gill is secondary to their relatively large size, disposition, and characteristic extremely low ionic fluxes (Evans, 1979). Nevertheless the few published measurements of the TEP indicate that both Na and Cl are maintained out of electrochemical equilibrium (Evans, 1980). We have recently found that prenatal "pups" of the spiny dogfish (*Squalus acanthias*) are plentiful and easy to handle and display the hallmarks of adult elasmobranch osmoregulation (Kormanik and Evans, 1978; Evans and Mansberger, 1979; Evans and Oikari, 1980). Importantly we have found that the head can be easily perfused and that it maintains hemodynamic stability for 2–3 hours (Evans and Claiborne, 1982). It is hoped that this preparation will allow a more careful dissection of any salt transport mechanisms which may reside in the elasmobranch branchial epithelium.

Conclusions

It should be obvious from this rather cursory review that substantial strides have been made in the elucidation of the transport parameters of the fish gill, due in no small part to the use of various "pieces" of the whole animal. To a considerable extent the techniques have been complementary with data from one system corroborating, but expanding, data from another system. However, each of the approaches has its advantages and disadvantages, which must be appreciated and accounted for. It is also obvious that, per usual, the number of species of fishes which have been examined is vanishingly small, and no single species has been examined utilizing all of the techniques described in this review. It is therefore appropriate to suggest that more species should be examined and that more investigators should use a variety of techniques, rather than a single method of approach.

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